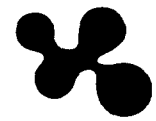
New Dietary Ingredient Notification for 7-Hydroxymatairesinol (HMR) Potassium Acetate Complex



Submitted by:

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In accordance with the Dietary Supplement Health and Education Act of 1994 (DSHEA), 21 U.S.C. § 350b (a) (2), and with final regulations published in Federal Register 49886-49892), 21 C.F.R. § 190.6 "Requirement for Premarket Notification", the following information is submitted by Hormos Medical Corporation (referred later to as to "Hormos") in support of a New Dietary Ingredient Notification for 7-hydroxymatairesinol.

Hormos intends to market 7-hydroxymatairesinol as a dietary supplement in the United States. As per the statutes of the DSHEA, 21 U.S.C. § 350b (a) (2), Hormos Medical Corporation will not introduce, market, distribute or sell 7-hydroxymatairesinol until at least 75 days following official acknowledgement of the receipt of this notification by the U.S. Food and Drug Agency (FDA).

SECTION 1

The name and complete address of the manufacturer of the dietary supplement that contains the dietary ingredient, or the dietary ingredient.

The manufacturer of the dietary ingredient will be:

Hormos Medical Corporation. Ltd.
Pharmacity
Itäinen Pitkäkatu 4B
FIN-20520 Turku
Finland

Attention: Mr. Lars Pellas
Vice President, Corporate Development

SECTION 2

2.1 The name of the dietary ingredient.

The new dietary ingredient is 7-hydroxymatairesinol (HMR), a dibenzylbutyrolactone plant lignan containing a 2,3-dibenzylbutane skeleton. Thus, HMR is chemically a close relative to matairesinol, a lignan present at high concentration in flaxseed. HMR is found at relatively high concentrations in the heartwood of branches and knots of Norway spruce (*Picea abies,* [L.] H. Karst.) trees. In addition, detectable levels are present in sesame seeds. Hormos Medical Corporation intends to manufacture HMR as a potassium acetate complex. HMR is a near white powder. Because of the chiral carbon at position 7, HMR is composed of 2 stereoisomers (allo-HMR and HMR) that are present in an approximate 1:9 ratio within the HMR potassium acetate preparation. HMR and its potassium acetate complex are slightly soluble in water and soluble in acetone, aqueous ethanol, methanol, acetonitrile, DMSO, PEG, and corn oil.

[Paragraph redacted - considered to be trade secret information]

[Paragraph redacted - considered to be trade secret information]

SECTION 3

Description of the dietary supplement or dietary supplements that contain the dietary ingredient including (i) the level of dietary ingredient in the dietary supplement, and (ii) the conditions of use recommended or suggested in the labeling of the dietary supplement, or if no conditions of use are recommended or suggested in the labeling of the dietary supplement, the ordinary conditions of use of the supplement.

3.1 Description of the dietary supplement

7-Hydroxymatairesinol potassium acetate complex (HMR potassium acetate complex) will be marketed for use in products meeting the definition of "dietary supplement" in section 201 (ff) of the Federal Food, Drug, and Cosmetic Act. The HMR-potassium acetate complex will be clearly labeled and promoted as a dietary supplement. HMR-potassium acetate complex will be sold in the form of oral capsules.

3.2 The level of dietary ingredient in the dietary supplement

An oral capsule will contain up to 50 mg of HMR, corresponding to approximately 72 mg of HMR potassium acetate complex.

3.3 Recommendations for the use of HMR potassium acetate dietary supplement

Consumption of 1 capsule per day will be suggested or recommended, resulting in a recommended maximum daily consumption of 50 mg HMR/day, or approximately 1 mg/kg body weight/day for a 50 kg person.

Consumption of the HMR-potassium acetate complex is recommended for the use of adults only.

SECTION 4

The history of use or other evidence of safety establishing that the dietary ingredient, when used under the conditions recommended or suggested in the labeling of the dietary supplement, will reasonably be expected to be safe, including any citation to published articles or other evidence that is the basis on which the distributor or manufacturer has concluded that the dietary supplement will reasonably be expected to be safe.

The overall safety of HMR potassium acetate complex is supported by the results of pharmacology, toxicology and related testing, clinical studies in humans, and by data on other related plant lignans that are consumed directly in conventional food or which are marketed as dietary supplements.

4.1 Safety pharmacology studies

The results of unpublished standard general pharmacology studies demonstrate that HMR does not have pharmacological activity [1-10]. Specifically, HMR did not affect mean arterial pressure and heart rate in rats administered doses of up to 30 mg/kg body weight by i.v. injection[1]. There were no effects of HMR on pentobarbital-induced sleeping times in rats dosed by oral gavage at up to 100 mg/kg body weight[2]. Similarly, at this dose level HMR did not alter motor co-ordination[3], spontaneous motor activity[4], and gastrointestinal transit time in mice[5], or rectal temperatures[6], pain thresholds[7], and kidney function in rats[8]. There were no overt effects of HMR, administered orally at doses of up to 1,000 mg/kg body weight, in male NMRI mice in the Modified Irwin Screen test[9]. In telemetered dogs treated by oral gavage with single HMR doses of 2, 20, and 200 mg/kg body weight, there were no obvious effects on respiratory rate, tidal volume, or minute volume[10]. Electrocardiograms showed no effects of HMR on P-wave amplitude, P-wave duration, P-Q interval, QRS interval, or Q-T interval[10].

4.2 Toxicity studies in rats and dogs

4.2.1 Toxicity studies up to 28 days

The toxicity of HMR has been extensively studied in animals. One acute toxicity study[11], 2 subchronic toxicity studies (28-, and 90-day studies) [12, 13], and a teratology study[14] have been performed in the rat. A 15-day [15] and a 28-day study [16] have also been conducted in the beagle dog.

In an unpublished acute toxicity study[11] single gavage doses of 1,000 and 2,000 mg HMR/kg body weight were administered to groups of male and female rats. Signs of toxicity were then monitored for 14 days. The test was performed in accordance with Organization for Economic Co-operation and Development (OECD) guidelines for acute

oral toxicity testing. There were no deaths or other signs of toxicity reported. Similarly, there was no effect of treatment on macroscopic or microscopic observations. The authors concluded that the minimal lethal dose was above 2,000 mg HMR/kg body weight.

In the 28-day subchronic rat study[12] HMR was administered by oral gavage to groups of 10 male and 10 female Sprague-Dawley rats at doses of 0, 239, 477, and 955 mg/kg body weight/day, and 0, 242, 483, and 967 mg/kg body weight/day for the first 18 and the second 15 days, respectively. An additional 3 animals/sex/group were included for toxicokinetic analyses. The toxicokinetic bioanalysis of plasma samples demonstrated that rats were dose-dependently exposed to the test article (Table 1). There were no treatment-related effects on body weight, body weight gains, food consumption, organ weights, or on hematological, clinical chemistry, and urinalysis parameters. Four high-dose females, 1 high-dose male, and 1 mid-dose female died likely due to a relatively large volume of a bolus dose combined with a thick consistency of the test article formulation. There was no effect of treatment on the incidence of findings at pathological examination, except for the finding of a large thyroid in 1 high-dose male. Histopathological examination indicated the presence of slight follicular cell hypertrophy in high-dose males. The relationship of this finding to treatment was unknown; however, since no indications of thyroid follicular cell hypertrophy were seen in a 90-day study[14] in which rats were treated with HMR at dose levels of up to 2,600 mg/kg body weight/day, the finding in the 28-day study was considered incidental. In any case, oral gavage administration of HMR at doses of up to 967 mg/kg body weight/day for 28 days was well tolerated without overt toxicity.

Table 1. Toxicokinetic summary from the 28 day subchronic toxicity study with HMR in rats[12]

Dose (mg/kg/day)	Exp. Day	Cmax (ng/mL)*		AUC(0-8h) (h.ng /mL)*		Tmax (h)*	
		male	female	male	female	male	female
250	1	660	422	1897	1240	0.5	0.5
	28	576	461	2466	1396	6	0.5
500	1	1760	1800	6160	3978	1	0.5
	28	1800	700	3053	1964	1	0.5
1000	1	4600	1840	16138	7627	2	2
	28	3020	11300	10621	19412	0.5	8

* group mean values

In a preliminary "Maximum Tolerated Dose" study[15] HMR was administered in capsule form to 1 male and 1 female beagle dog at escalating doses of 0, 200 (days 1-4), 500 (days 5-7), and 700 (days 8-11) mg/kg body weight/day for 11 days. At the end of dosing, the control animals were dosed at 700 mg/kg body weight/day for 15 days. A second formulation of HMR was tested in capsule form at a dose of 636 mg/kg body weight/day for 5 days in one female and one male dog used in the MTD phase of the study. The MTD phase of the study demonstrated that 700 mg/kg body weight/day was well tolerated. Dosing at 700 mg/kg body weight/day for 15 days produced no treatment-related effects on clinical signs, mortality, body weight, body weight gain, food

consumption, hematology or clinical chemistry parameters, or on the results of organ weight assessment and macroscopic examinations.

HMR was subsequently tested in groups of 3 male and 3 female beagle dogs by daily administration in capsule form at doses of 0, 146-149, 341-347, and 682-685 mg/kg body weight/day for 28 days[16]. There were no treatment-related effects on mortality, clinical signs, hematological or clinical chemistry parameters, urine analysis, organ weight data, or on the results of electrocardiographic examinations. The group mean body weight of the high-dose males was found to be consistently lower than controls at pre-dose and throughout the study period. Similarly, in high-dose animals, food consumption appeared slightly decreased compared to controls. Food consumption did increase in these groups as the study progressed. The mean absolute and relative uterine weights appeared increased in dosed females; however, given the lack of dose-response or of histopathological correlates, and noting that all of the values were within the historical control ranges, this finding was not considered to be related to HMR treatment. Histopathological examination revealed a single healing erosion in the ileum of 1 high-dose female dog (correlating to a red, depressed area noted at gross necropsy) and of follicular cell hypertrophy in 1 male and 1 female of the high-dose groups. Due to the small number of animals used in this study, the relationship of these macroscopic and histopathological findings cannot be ascribed to treatment, but neither can a treatment-related effect be discounted.

4.2.2 Toxicity studies up to 90 days

In an unpublished subchronic toxicity study[13] groups of 20 male and 20 female Wistar rats were fed HMR in the diet at concentrations of 0, 0.25%, 1.0%, and 4.0% for 13-weeks. These dietary concentrations resulted in HMR intakes of approximately 0, 160, 640, and 2,600 mg/kg body weight/day. There were no effects of HMR treatment on mortality, neurobehavioral observations, motor assessment results, ophthalmoscopic examinations, urinalysis parameters, sperm analysis, or on the results of the macroscopic examinations. Histopathological examination revealed a decreased incidence of hyaline droplet nephropathy, a beneficial effect, in high-dose males.

Male rats dosed at 4.0% in the diet showed decreased body weights throughout the study period. In mid-dose males and in mid- and high-dose females, this effect occurred only in the first few weeks of the study. In both sexes treated at the high-dose, there was an increase in the number of animals with sparsely haired skin. Hematological analyses at the end of the study revealed increased thrombocytes in high-dose females and increased WBC count and absolute neutrophil count in high-dose males. Several clinical chemistry changes were noted, including: decreased cholesterol and fasting glucose in high-dose males, increased albumin and A/G ratio in high-dose males, increased GGT in high-dose females, decreased triglycerides in treated males, decreased phospholipids in mid- and high-dose males, and increased total bilirubin in mid- and high-dose males. The clinical chemistry changes, in particular the findings of reduced cholesterol, triglycerides, and phospholipids, have been noted in response to dietary administration of high fiber

concentrations that are metabolized by gut microflora (as with HMR). This metabolism results in production of short-chain fatty acids that stimulate hepatic and peripheral metabolism of carbohydrates and fats [17, 18,19] .

Several organ weight changes were reported in the treated groups. In treated males and in high-dose females, the relative weight of the filled cecum was increased. Similarly, both the absolute and relative weights of the empty cecum were increased in males at the top 2 dose levels and in high-dose females. This effect is a well-known physiological response to high-dietary concentrations of certain substances that alter the osmotic loading of the lower gut, and as such is not considered adverse [20,21,22, 23]. In high-dose males, the relative kidney and testes weights were increased, and the relative weight of the adrenal and the absolute brain weight were decreased, in comparison to controls.

Absolute ovarian weights were statistically significantly decreased in all treatment groups (including low-dose; 160 mg/kg/day). Statistically significant decreases in relative ovary weights were confined to the mid- (640 mg/kg/day) and high-dose (2600 mg/kg/day) groups. The absolute weight in the low-dose group was only marginally decreased ($0.01 < p < 0.05$) and this decline was just outside the "low mean" (*i.e.* 0.069 grams *versus* 0.070 grams) of the historical control range reported for the 13 week studies conducted in the laboratory, and was not associated with changes in estrous cycle length, an effect occurring only at the high-dose (vaginal smears taken during the last 3 weeks prior to sacrifice demonstrated that the number of high-dose females with a maximum estrous cycle length of 4 days was decreased, while those with a maximum cycle length of 5 days was increased, in comparison to controls). In addition, the relative ovarian weight at the 160 mg/kg/day dose level was not decreased to a statistically significant extent, and was within the historical control range for this parameter. Given also the lack of histopathological effects on the ovaries (or any other organs) up to the highest dose tested (more than 15-fold higher than the low-dose level at 160 mg/kg/day), the 160 mg/kg bw/day dose level is considered a No Observable Adverse Effect Level (NOAEL). Similarly, the study's authors considered the 160 mg/kg/day dose (0.25% in the diet) to be the "No Observable Effect Level" (NOEL).

The effects of HMR on ovarian weights (mid and high-dose) and on estrous cycle length (high-dose only) could be indicative of a very weak antiestrogenic effect consistent with stronger such effects known for many other phytoestrogens, including soy isoflavones and secoisolariciresinol diglycoside (SDG) derived from flaxseed[24,25,26]. These effects may be related to the metabolism of HMR to enterolactone[25]. Minor changes in ovarian weights and in estrous cycle length are not adverse and are not indicative of strong estrogenic potency. The potential estrogenic effects of HMR were evaluated in an unpublished study[27] on the ability of HMR to competitively inhibit the *in vitro* binding of a fluorescein labeled estrogen receptor ligand (ES2) to estrogen receptors α and β. Competitive binding was compared to the standard of 17β-estradiol. HMR was found not to competitively bind with ES2 for either estrogen receptors α or β. The maximum concentration of HMR tested in this assay was 10,000 nM. In contrast, the intestinal bacterial metabolite of HMR, enterolactone, has a weak binding affinity to the α and β estrogen receptors with an $IC_{50} > 10$ uM.

4.2.2.1 Ovarian weight effect safety margins: HMR level comparison

In the 13-week subchronic feeding study[13], there were no separate groups for toxicokinetic exposure analysis. However, to determine safety margins between the 160 mg/kg/day NOAEL for the decreased relative ovarian weights reported in rats, Hormos has conducted additional bioanalyses of terminal plasma samples from the 13 week subchronic toxicity study of HMR [a separate bioanalytical report ref.[28]]. In rats receiving the low-dose (160 mg/kg/day) feed, the plasma level of unconjugated HMR was 74 ng/ml (average from female and male data). In human subjects receiving HMR at daily doses of 315 mg for 4 wk, the plasma overnight steady state unconjugated HMR level was 3.7 ng/mL after 28 days of continuous dosing[29]. Thus, assuming a 50 mg/day continuous dosing schedule, plasma steady state levels are estimated to be 0.59 mg/mL (*i.e.*, (50 mg/315mg) x 3.7 ng/mL). As a result, by comparing the steady state concentrations in the subchronic toxicity study (at 160 mg/kg/day) and associated with 50 mg human daily consumption, there exists a 125-fold safety margin (*i.e.*, 74 ng/mL in the rat study compared to 0.59 mg/mL estimated to be associated with consumption of 50 mg HMR per day under the intended conditions of use).

Furthermore, the estimated HMR AUC value for the 160 mg/kg/day dose (extrapolated from the 250 mg/kg/day dose in the 28 day toxicity study) is approximately 1120 h.ng/mL. From the HMR single dose human clinical trial[30], it may be extrapolated (from 30 mg and 100 mg dose level data) that a 50 mg single HMR dose results in an AUC of 25 h.ng/ml. As a result, there is approximately a 45 –fold safety margin based on HMR AUC values.

4.2.2.2 Ovarian weight effect safety margins; Enterolactone metabolite comparison

In addition to the large safety margins, based on HMR plasma levels between the NOAEL for decreased relative ovarian weights reported in the 13 week rat study and 50 mg HMR human daily consumptions, there are large safety margins associated with plasma levels of the intestinal metabolite of HMR, enterolactone. This is particularly relevant since enterolactone has been shown to have a low affinity towards estrogen receptors **α and β** [27]. Since enterolactone is metabolically fermented from the parent molecule by intestinal microbes, the plasma profile is quite different from the parent molecule (HMR). Enterolactone appears in the plasma slowly and with no sharp C_{max} value (exemplary plasma curves are available within the human repeated dose clinical trial report[29]). Detectable free enterolactone occurs, at minute levels, only at high HMR dosages (> 300 mg) and usually 24-48 hrs after dosing (see ref [30], appendix I, bioanalytical study report). For this reason we have no data to compare AUC values for enterolactone. However, the steady-state unconjugated enterolactone levels were measured in the 13-week subchronic study in the low dose rats and were found to be 62 ng/mL[28]. In the 4-week human clinical trial, 28-day continuous dosing at 315 mg/day did not result in any measurable unconjugated enterolactone concentration in the plasma (quantitation limit of 0.4 ng/mL). Given the 0.4 ng/ml quantitation limit, and based on linear extrapolation from the 315 mg/day dose in the human trial, 50 mg daily doses to humans would be expected to produce plasma enterolactone concentrations of < 0.06

ng/mL (*i*.e., [50 mg/315 mg] x < 0.4 ng/ml). As a result, by comparing the steady state safety margins for enterolactone exposure in the 13-week rat study (unconjugated enterolactone in plasma at 62 ng/ml) and the 4-wk human study (unconjugated enterolactone in plasma of < 0.06 ng/ml, day 29 value), the safety margin appears to be at least 1033-fold. Further details are provided in Table 2.

Table 2. Safety margins for the rat ovarian weight effect

Parameter	Rat 13 -week study (160 mg/kg/day)	Human (50 mg/day)	Safety margin (fold)
steady state, HMR (ng/mL)	74	0.59[a]	125 x
steady state, enterolactone (ng/mL)	62	<0.06[b]	>1033 x
AUC, HMR h.ng/mL	1120[c]	25[d]	45 x

[a] Extrapolated (linear) for 50 mg dose from the 4-wk repeated dose human clinical trial [29]: the overnight residual unconjugated HMR concentration (3.7 ng/mL) after 28 days of continuous HMR administration at 315 mg/day.

[b] Extrapolated (linear) for 50 mg dose from the 4-wk repeated dose human clinical trial [29]: the overnight residual unconjugated enterolactone concentration (< 0.4 ng / mL) after 28 days of continuous HMR at 315 mg/day.

[c] Extrapolated for 160 mg/kg/day dose from the measured AUC value after 250 mg/kg/day HMR (1749 h*ng/mL; mean AUC for female and male rats, day 1 and day 28 values averaged; [12]).

[d] Extrapolated from 30 and 100 mg single dose human data [30].

4.2.2.3 The HMR effect on ovaries is antiestrogenic

HMR has been studied in the immature rat uterus weight test (50 mg/kg/day for 7 days) without indication of estrogenic activity[31]. On the other hand, chronic treatment with low dose antiestrogens has also resulted in decreased ovarian weight in rats[32,33]. Therefore, it is likely that a mild antiestrogenic effect of enterolactone is responsible for the observed decreased ovarian weights in the HMR exposed rats.

In conclusion, the observed decrease in the relative ovarian weight in rats fed more than 160 mg/kg/day HMR might represent a mild phytoestrogen response (antiestrogenic effect) likely resulting indirectly from exposure to enterolactone rather than being a direct effect of HMR. Due to the large safety margins in the enterolactone levels achieved in the rat study compared to humans exposed under the intended conditions of use (minimum of 1033x), there is no risk of endocrine effects at the proposed level of HMR use in humans.

4.2.3 Teratogenic potential

The teratogenic potential of HMR has been evaluated in an unpublished prenatal developmental toxicity study[14] HMR was administered in the diet to groups of 24 mated female Wistar outbred rats at concentrations of 0, 0.25%, 1.0%, and 4.0% from gestational day 0 (fertilization) through until gestational day 21. These dietary concentrations equated to HMR intakes of approximately 0, 140-180, 460-740, and 1,190-2,930 mg/kg /day in the control through high-dose groups, respectively. At

Caesarean section dams and fetuses were macroscopically examined and the fetuses, placental material, reproductive organs, and the full and empty cecum weighed. Fetuses were further evaluated for both visceral and skeletal abnormalities.

Thirteen of the high-dose animals (out of 24) were sacrificed due to the fact that they either did not eat or ate less than 4 g of food per day. There were no differences in the clinical signs between the treated and control groups. Both body weight and food consumption were significantly decreased in high-dose animals from gestational day 3 through until Caesarean section. This was most probably related to poor palatability of the diet. Necropsy of the dams did not reveal any treatment-related pathological changes. The absolute and relative weights of the full cecum were increased in the high-dose group while the absolute and relative weights of the empty cecum were increased in both the mid- and high-dose groups. There were no pathological changes associated with the increased cecal weights. The study authors concluded that the maternal NOEL was at 1.0% in the diet or at least 460 mg/kg body weight/day based on a decreased body weight at the top dose.

There were no effects of treatment on reproductive indices, including: female fecundity, number of corpora leutea, number of implantation sites, number of live fetuses, number of early and late resorptions, sex-ratio, and amount of pre- and post-implantation loss. External evaluation of the fetuses and placental material revealed no effects of treatment. The weights of the fetal females and of all fetuses combined were decreased from dams of the high-dose group. There were no treatment-related effects on the incidence of either visceral or skeletal abnormalities. The incidence of kinked ureter was increased in fetuses from the mid- and high-dose dams; however, this particular finding is generally considered a variation [34] and essentially harmless [35]. The study authors concluded that the NOEL for developmental toxicity was at least 4.0% in the diet (at least 1,190 mg/kg body weight/day), the highest dose tested in the study.

In conclusion, HMR is not expected to pose any teratogenic/developmental toxicity risk.

4.2.4 Genotoxic potential

The genotoxicity of HMR was evaluated in 3 unpublished studies sponsored by Hormos Nutraceutical Oy Ltd., including an Ames bacterial mutagenicity assay[36], an *in vitro* chromosome aberration assay[37] and an *in vivo* rat bone marrow micronucleus study[38]. All 3 of these studies were conducted according to OECD protocols and were consistent with U.S. FDA Redbook guidelines.

4.2.4.1 *In vitro* mutagenicity in *S. typhimurium*

In the Ames assay[36], the test substance in dimethylsulfoxide (DMSO) was not toxic to *Salmonella typhimurium* strain TA100 at dose levels of 1.6, 8, 40, 200, 1,000, or 5,000 µg/plate. Similarly, HMR was not toxic to *Salmonella typhimurium* strains TA98, TA102, TA1535, or TA1537 at doses of 5,000 µg/plate. Following incubation with

HMR, both in the absence and in the presence of an exogenous source of metabolic activation (Aroclor 1254-induced liver post-mitochondrial fraction (S9) from Sprague-Dawley rats), there were no biologically or statistically significant increases in the number of revertant colonies in comparison to solvent treated controls.

4.2.4.2 *In vitro* genotoxicity in cultured Chinese hamster ovary cells

HMR was tested in an *in vitro* cytogenetic assay using Chinese hamster ovary (CHO) cells[37]. In one set of experiments, HMR was incubated, either in the presence or absence of an exogenous source of metabolic activation (rat S9), for 3 hours followed by a 17-hour recovery period. Concentrations tested ranged from 0 to 1083 µg/ml (without S9) and from 0 to 1227 µg/ml (with S9). At the highest concentrations tested in each case there was a significant increase in the number of cells with chromosomal aberrations compared to solvent treated controls. However, at these concentrations, cell numbers were decreased by 51 and 60% in the presence and absence of S9, respectively (the summary of the data is presented in Table 2). This indicates that concentrations associated with chromosomal aberrations were also associated with significant cytotoxicity. In a second series of experiments, treatment was repeated in the absence and in the presence of S9 for 3 hours plus a 17-hour recovery period. As in the first experiment, the highest concentrations tested (862.4 µg/ml and 1253 µg/ml in the absence and presence of S9, respectively) were associated with an increase in the frequencies of structural aberrations. However, as before, at these concentrations, a 51 to 59% reduction in cell survival was reported (Table 3).

Table 3. Result of the performed clastogenicity study in CHO cells

Exp		Test article (µg/mL)	Aberration score*	cell number reduction (% from control)
I	- S9	neg. control	2 / 2	0
	- S9	838	3 / 3	35.4
	- S9	1083	49 / 42	59.5
	- S9	positive control	52 / 50	ND
	+ S9	neg. control	2 / 2	0
	+ S9	785	2 / 1	21.7
	+ S9	1227	17 / 13	51.0
	+ S9	positive control	81 / 80	ND
II	- S9	control	5 / 3	100
	- S9	667.3	5 / 3	34.2
	- S9	862.4	39 / 35	50.5
	- S9	positive control	36 / 33	ND
	+ S9	neg. control	4 / 4	0
	+ S9	769.4	3 / 3	36.6
	+ S9	1253	37 / 36	58.6
	+ S9	positive control	142 / 141	ND

* including / excluding gaps, ND = not determined

In conclusion, a threshold clastogenic concentration exists above 839 μg/mL of HMR. As concluded also by the study authors, an at least 50% reduction in cell number is associated with induction of chromosome aberrations by HMR.

4.2.4.2.1 HMR causes membranous, cytotoxic damage in CHO cells.

An *in vitro* acute toxicity study of HMR performed in CHO cells indicates[39] that HMR causes a cytotoxic effect at a concentration of 1000 μg/mL and above.

As measured immediately after the 3 hr pulse HMR treatment (treatment regimen adopted from the chromosome aberration study protocol), there is a considerable increase in acute toxicity at concentrations above 1100 μg/mL. Toxicity is demonstrated by an increase in adenylate kinase (AK) activity. Release of AK is indicative of the loss of the integrity of the cell membrane and as such provides a good biomarker of cytotoxicity. The increases in AK activity following 3 hour pulse treatment with HMR are shown in Figure 1:

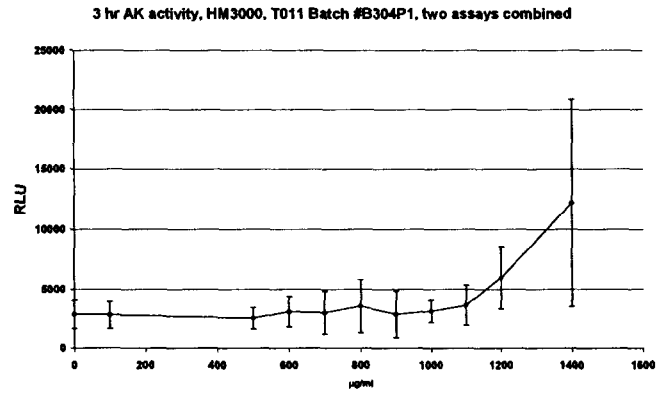

Figure 1, RLU= relative luminescence unit

After the 3 hr test article (HMR) treatment, there is additional toxicity following a 17 hr recovery period (regimen adopted from the original CHO chromosome aberration assay[37]). Following a 17 hr recovery period, AK released from the CHO cells was increased at HMR concentrations greater than 1000 μg/mL. These results are presented graphically in Figure 2.

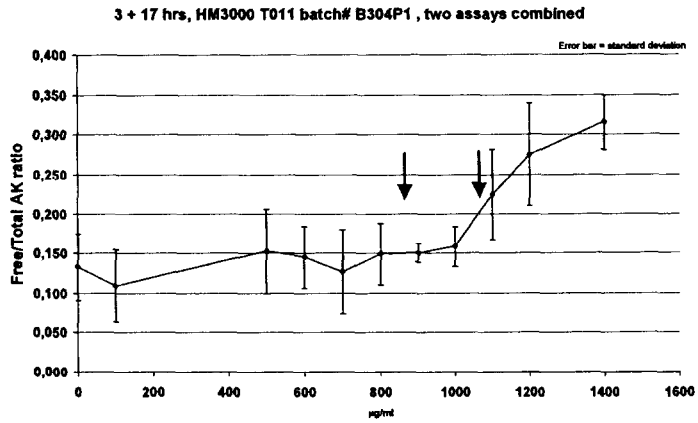

Figure 2

The acute toxicity study, using the treatment schedule from the original chromosome aberration study[37], verifies that HMR has an acute cytotoxic effect at concentrations of approximately 1000 µg/mL and higher. In the original study, the chromosome aberrations occurred at concentrations of 1083 and 862 µg/mL (shown by arrows in the Figure 2). As a result, the clastogenic effect appears within the cytotoxicity range of HMR.

Cytotoxicity has been addressed by several authors as a phenomenon that may be causally associated with the induction of chromosomal damage in mammalian cell tests[40,41]. Considering that the clastogenic effect of HMR appears to have a threshold and that considerable cytotoxicity was induced at this threshold concentration, the chromosome aberration result appears to be secondary to the cytotoxic effect, and not an indication of direct genotoxicity. The negative Amestest also supports this conclusion[36].

It is generally assumed that DNA damage constitutes the primary signal for the induction of apoptosis, or programmed cell death, in response to DNA damaging agents, such as alkylating agents[42]. Therefore, we also investigated the ability of HMR to induce apoptosis in the setting used above to estimate chromosomal damage in CHO cells[39]. Caspase-3 is a downstream aspartic protease, and a commonly used general marker to measure the level of apoptosis in *in vitro* mammalian cell cultures. Caspase-3 activities remained unaffected by HMR concentrations of up to 1400 µg/mL. This indicates that the observed cytotoxicity as measured by the AK release was not due to programmed cell death (that could be secondary of DNA damage), but is likely to be a direct toxic effect of HMR on cell membranes, leading to secondary effect such as leakage of intracellular constituents outside the cell, and other cellular perturbations including damage to DNA.

4.2.4.3 Mutagenic potential of HMR *in vivo*

In addition to the data from the acute toxicity study in CHO cells showing that HMR only produces chromosome aberrations at concentrations producing concomitant cytotoxicity

(as measured by AK), negative data (coupled with new toxicokinetic data; shown below) from the rat micronucleus study[38] demonstrates that HMR has no genotoxic potential.

In the *in vivo* rat bone marrow micronucleus assay, groups of 6 male Charles River CD rats were treated with HMR by corn oil gavage at doses of 0, 500, 1,000 and 2,000 mg/kg body weight once daily for 2 consecutive days[38]. Doses were established on the basis of an initial toxicity study in 3 male and 3 female rats. The rats were killed 24 hours after the second administration. Treatment with HMR did not increase the group mean ratios of polychromatic:normochromatic erythrocytes above the values reported for the negative controls or in comparison to historical control ranges. Similarly, the frequencies of micronucleated polychromatic erythrocytes were unaffected by treatment. The positive control, cyclophosphamide, administered by gavage in saline at a dose of 20 mg/kg (single dose) produced the expected increase in micronucleated polychromatic erythrocytes. Based on these results, the study authors concluded that HMR did not induce genotoxic effects in this assay system.

In the previous dossier submission there were no toxicokinetic data presented to support an adequate estimation of systemic exposure to HMR and metabolite(s). The toxicokinetic samples of this study have now been analysed and reported separately[43] . The treatment resulted in peak levels of 1147 ng/mL of unconjugated HMR in plasma. While no area under the plasma concentration curve (AUC) data were available from the micronucleus study, in the 28 day toxicity study in rats[12], a daily dose of 1000 mg/kg resulted in an AUC value of approximately 13 400 h.ng/mL (average for day 1 and day 28 AUC -values in male and female rats). As a result, based on linear extrapolation, the AUC achieved in the rat micronucleus study is estimated to be 26 800 h.ng/mL at the 2000 mg/kg dose.

From the HMRlignan™ single-dose human clinical trial[30] it was extrapolated (from 30 mg and 100 mg dose level data) that a 50 mg single HMR dose results in a peak level of approximately 11 ng/mL unconjugated HMR in plasma and in an AUC of 25 h.ng/mL. As a result, based on plasma C_{max} values in the rat micronucleus study (1147 ng/mL – measured), the systemic exposure in the negative micronucleus assay was 104-fold greater than the maximum systemic human exposures likely to occur under the intended conditions of use. Based on the AUC data (26 800 h.ng/mL estimated in rats and 25 h.ng/mL estimated in humans), the systemic exposures achieved in the negative micronucleus assay were 1072-fold greater as compared to human systemic exposure.

In conclusion, the rat micronucleus study shows that HMR is not genotoxic *in vivo*. Also, the toxicokinetic data demonstrates that the systemic exposures achieved in the micronucleus test were adequate and many multiples of maximum human exposures under the intended conditions of use. A summary of the toxicokinetic data is presented in Table 4.

Table 4. Systemic Exposures Achieved in the Rat Micronucleus Study in Comparison to Human Systemic Exposures Associated with Dosing at 50 mg/day

Parameter	Systemic HMR exposure in the rat micronucleus study at 2,000 mg/kg/day	Systemic HMR exposures estimated to occur at human dose of 50 mg/day
C_{max} (ng/mL)	1147	11[b]
AUC (h*ng/mL)	26 800[a]	25[b]

[a] Extrapolated from the toxicokinetic data from the rat 28 day toxicity study [12], 1000 mg/kg HMR dose level data.

[b] Extrapolated from 30 or 100 mg HMR dose level data in a human single dose clinical trial [30]

4.2.4.4 HMR has no effect on a marker of genotoxicity in humans

A marker for oxidative DNA damage, 8-hydroxy-2′-deoxyguoanosine (8-OHdG) has been measured from the peripheral white blood cells in human subjects exposed to HMR up to 1350 mg/day for 4 weeks[29]. The level of 8-OHdG has been linked to oxidative DNA damage[44] and elevated 8-OHdG levels have been measured in workers occupationally exposed to asbestos or styrene[45,46]. In the 4-week clinical trial[29], repeated dosing with HMR at exposure levels 27-fold greater (*i.e.*, 1350 mg/day *versus* 50 mg/day under the intended conditions of use) was devoid of any effect on 8-OHdG levels. This observation further supports the conclusion that HMR has no genotoxic potential under the intended conditions of use.

4.2.4.5 Lignans closely related to HMR are not genotoxic

Finally, the lack of genotoxic potential of HMR is underscored by the genetic toxicity data available for the closely related lignans matairesinol, secoisolariciresinol as well as their bacterial metabolite enterolactone[47]. In this study[47], these compounds were tested at 200 µM in cell free systems (microtubule assembly tests) and at 100 µM in Chinese hamster V79 cells (cytoplasmic microtubule complex; mitotic arrest; micronuclei and mutagenicity at HPRT-locus). None of the tested lignans or enterolactone was reported to show evidence of genotoxicity for the endpoints and concentrations studied.

In conclusion, chronic consumption of HMR at the maximum intended dose level of 50 mg/day would not pose a genotoxic risk. The bases for this conclusion include: a) the *in vitro* cytotoxicity study confirms that cytotoxicity can account for the observed increase in aberrations in the CHO assay; b) the rat micronucleus study produced negative results and the toxicokinetic data demonstrate systemic exposures that were many multiples of the systemic human exposures expected under the intended conditions of use; c) HMR was not mutagenic in the Ames test; d) the measurement of the putative clinical genotoxic marker 8-OHdG did not indicate any increase in oxidative DNA damage in human subjects receiving 1350 mg HMR per day for 4 weeks; and, e) data showing that closely related lignans are not genotoxic.

4.2.5 Additional *in vivo* and *in vitro* studies with HMR

The metabolism of HMR was evaluated in a published study[31] in rats. Rats were administered HMR by oral gavage at doses of 3, 15, 25, and 50 mg/kg bw on 2 consecutive days during which time they were housed in metabolic cages and urine collected for gas chromatographic analysis of total concentration of HMR and metabolites. Enterolactone was the major metabolite identified reaching levels in the urine that were more than 8-fold those found in controls. Other metabolites detected included hydroxyenterolactone, α-conidendrin, conidendric acid, enterodiol, allo-HMR, and unchanged HMR. No analysis of potential fecal metabolites was conducted. All of these minor metabolites, including unchanged HMR, were at concentrations many-fold (usually greater than 10-fold) lower than the primary metabolite enterolactone.

In another unpublished study[48] designed to evaluate the absorption and tissue distribution of HMR, tritiated-HMR was administered to groups of 2 male and 2 female Sprague – Dawley rats by both oral gavage and intravenous injection. Following oral administration at 250 mg/kg bw, measures of total radioactivity indicated that HMR was well absorbed, with peak plasma concentrations occurring approximately 1 hour post-dosing. Non-volatile radioactivity was reported to be well distributed to body tissues with the highest concentrations found in the gastrointestinal and urinary systems. Rapid clearance of total radioactivity (concentrations in tissues and plasma less than 10% of peak values at 24 hours post-dose) was reported, indicative of rapid excretion in the urine. By comparing AUC values for plasma concentrations from the oral study with AUC values obtained following dosing *via* intravenous injection (25 mg/kg bw), the authors concluded that about 56% of the initial radiolabeled dose was absorbed following oral administration. In addition, it was apparent that the sites of the radiolabel in parent HMR were stable as there was very minimal exchange of radiolabel with body water.

The results of the *in vivo* metabolic studies are complimented by an *in vitro* study[49] in which HMR, as the individual diastereoisomers HMR and allo-HMR, was incubated in the presence of human or rat liver homogenate preparations. Both HMR and allo-HMR were rapidly metabolized by the human liver homogenate (*i.e.*, about 80%-90% cleared within 60 minutes), with glucuronidation comprising the most significant biotransformation process, accounting for more than 95% of the metabolites generated. Compared to the human liver homogenate, rat liver preparations were much less efficient in clearing either HMR isomer. Only about 30%-60% was cleared within 60 minutes by the rat liver preparation. The metabolite profile and the active biotransformation processes, however, were similar between the 2 species. In contrast to the results of the *in vivo* study in rats, enterodiol and enterolactone metabolites were not found to occur following incubation with either the human or rat liver homogenate *in vitro*. This result is expected given the recognized conversion of plant lignans into their mammalian counterparts (enterolactone and enterodiol) by way of facultative intestinal microbes [50,51]

In addition to the preceding standard pre-clinical studies, Hormos Nutraceutical Oy Ltd. sponsored several specialized studies to investigate the antioxidant[52,53] and chemo-preventive nature of the compound [31,54,55].

The results of the unpublished investigations of the antioxidant properties of HMR, both *in vitro* and *in vivo*, showed that this substance was an effective antioxidant. *In vitro*[52], HMR: a) inhibited lipid peroxidation induced by tert-butylhydroxyperoxide (IC_{50} of 0.06 µmol/L), b) inhibited oxidation of human low-density lipoprotein (LDL) by copper (IC_{50} of 6.7 nmol/mg LDL), c) inhibited LDL oxidation due to incorporation into LDL particle (IC_{50} of 130 nmol/mg LDL), d) scavenged superoxide anions produced by xanthine-xanthine oxidase (EC_{50} of 5.6 µmol/L), and e) scavenged peroxyl radicals generated by thermal decomposition of 2,2'-azobis(2-amidinopropane) hydrochloride (1 mole of peroxyl radical scavenged per 4 moles of HMR). Minor metabolites of HMR, including α-conidendrin and conidendric acid also showed significant antioxidant activity in the above *in vitro* assays.

In one of the *in vivo* antioxidant studies[53], weanling male Sprague-Dawley rats were fed a diet containing HMR to provide a dose of approximately 40 mg/kg bw/day for 5 months. Controls received diets not supplemented with HMR. Following the 5 month feeding period the animals were killed and liver, lung, and kidney tissues examined for signs of oxidative stress, specifically diene conjugation as an indication of lipid peroxidation. HMR had no effect on any measures of oxidative stress. In a second experiment, oxidative stress was induced experimentally in male mice through combined vitamin-E deficient diets and administration of carbon tetrachloride. Test diets contained either HMR (300 ppm or about 50 mg/kg body weight/day) or HMR in combination with α-tocopherol (restored to normal dietary levels). Diets were administered for up to 4 weeks. Oxidative stress was measured through analysis of the presence of thiobarbituate reactive substances (TBARS) in the liver. HMR alone and in combination was shown to substantially reduce the generation of TBARS (signs of oxidative stress). Also, HMR appeared to potentiate the antioxidant effects of vitamin E.

In a published study of the chemo-preventative effects of HMR[54], the inhibitory effects of HMR or rye bran on intestinal tumor development was tested in adenomatous polyposis colimultiple intestinal neoplasia $(Apc)^{Min}$ mice. HMR, along with inulin (2.5%), was administered to the male mice in a high fat diet, at a concentration of 200 ppm for a period of 5 to 6 weeks. Compared to the control diet (2.5% inulin, high fat diet), and compared to the control diet/rye bran combination, in HMR treated mice the mean number of adenomas in the small intestine was significantly lower (26.6 *versus* 39.6 and 36.0 in mice administered the control diet and the rye bran/control diet, respectively). HMR also appeared to normalize the levels of β-catenin concentrations within the adenomatous tissue. This was considered by the authors to indicate that the chemo-preventative effect of HMR was mediated through the Apc- β-catenin pathway. The authors did note that although there were no differences in body weight gain amongst the treatment and control groups, mice administered diet supplemented with HMR tended to have an increased amount of hair shedding.

Further chemo-preventative activity of HMR was demonstrated in an unpublished study in which, 3-days following injection of LNCaP prostate cancer cells into both flanks of male nude mice, treatment with HMR in the diet at concentrations of 0.15% and 0.3% for 9 weeks reportedly decreased mean tumor weight and serum concentrations of PSA[55]. Similarly, in a rat mammary tumor model in which mammary tumors were induced by 7,12-dimethylbenz[a]anthracene, treatment with HMR in the diet was reported to inhibit tumor growth at 15 mg/kg bw/day over a period of up to 17 weeks [31].

4.3 Human studies

In addition to the aforementioned preclinical safety data, 2 clinical trials have [29,30] been conducted to assess the tolerability, safety, and pharmacokinetics of HMR in human subjects.

4.3.1 Pharmacokinetics, safety and tolerability after single administration

The results of the single dose clinical trial have been presented in an unpublished report [30]. In this study, single doses (3, 10, 30, 100, 300, 600, and 1,200 mg) of HMR, or a placebo, were given to healthy male volunteers in capsule form. At the 4 lower dose levels (up to and including 100 mg), 3 subjects received HMR and 1 subject received placebo. At the 300 mg dose level, 8 subjects received HMR and 2 subjects received placebo. At the 2 highest dose levels, 5 subjects received HMR and 1 subject received the placebo. Plasma concentrations of unconjugated HMR, enterolactone, and other minor metabolites of HMR were measured 7, 3, and 1 day prior to HMR/placebo administration to establish baseline values. Following consumption of the capsule, blood was collected for analysis at 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 30, 48, and 72 hours post-dosing. LDL and DNA oxidation were measured prior to treatment and 2, 6, and 48 hours post-dosing. Safety was monitored through physical examination, clinical chemistry and hematological measurements, ECG recordings, and adverse event reporting (if any).

A tabular summary of the HMR pharmacokinetic results are presented in Table 5. The pharmacokinetic analysis revealed that peak concentrations of unconjugated HMR in plasma were reached 0.5 to 2.0 hours post-dosing, regardless of the dose administered. This result is consistent with the pharmacokinetic data obtained in rats. C_{max} values ranged from 0.29 ng/ml at the 3 mg dose level to 326.86 ng/ml at the 1,200 mg dose level. While the plasma levels of HMR increased with dose, the increase was often not linear and showed considerable inter-individual variation. Also, concentrations of HMR were detected in the plasma of the placebo group, and, in some cases, these concentrations exceeded those associated with the low dose of 3 mg. Elimination of HMR from plasma was rapid with $t_{1/2}$ values ranging from 2.6 to 5.1 hours.

Table 5. Pharmacokinetic summary of unconjugated HMR concentrations in plasma (mean ± SD) after single administration.

Dose group	C_{max} (ng/mL)	AUC_{0-24} (ng.h/mL)
placebo	0.5 ± 0.4	6.1 ± 5.3
3 mg	0.3 ± 0.2	2.0 ± 1.9
10 mg	1.0 ± 0.4	3.6 ± 2.5
30 mg	6.4 ± 1.8	14.4 ± 7.6
100 mg	22.0 ± 10.2	50.0 ± 23.0
300 mg	108 ± 57.6	181 ± 37.0
600 mg	90.6 ± 39.7	391 ± 226
1200 mg	327 ± 161.4	1312 ± 135

Plasma unconjugated enterolactone levels after single administration of HMR were generally low, and marginal increases (1 ng/mL or less) were observed 12 to 38 hrs after dosing at HMR single doses higher than 300 mg.

With respect to safety, the study authors concluded that HMR was well tolerated without any report of serious adverse events. There was no effect of HMR on clinical chemistry and hematological evaluations, vital signs, ECG recordings, or on the results of the physical examinations. There were no clear effects of HMR treatment on serum LDL oxidation products or the oxidation of DNA as measured by the levels of white blood cell concentration 8-OH-deoxyguanidine.

4.3.2 Pharmacokinetics, safety and tolerability after 4 week repeated dosing

A second tolerability, safety and kinetic study has been completed[29] involving multiple dosing of male volunteers with mild hypercholesterolemia. This study was designed to corroborate the safety and pharmacokinetics of HMR after continuous dosing at substantially high doses. Further, this study was a pilot study to investigate possible beneficial effect of HMR on lipoprotein oxidation products in mildly hypercholesterolemic men. In this study HMR was given to test subjects at total daily doses of 315 and 1350 mg (capsule administration, daily doses divided in three equal portions) for 4 weeks. Analysis of plasma HMR and metabolite levels confirmed the efficient absorption of HMR from the gastro-intestinal tract. Measurement of total enterolactone in plasma confirmed an active conversion of HMR into enterolactone and a sustained increase of the total plasma concentration of this metabolite during the treatment period. However, the measurement of unconjugated HMR and unconjugated enterolactone from the 315 mg/day kinetic subgroup (kinetic analysis on exp. days 1 and 29) revealed that only 1-2 % of total (unconjugated + glucuronide/sulphate conjugates) HMR in plasma is in unconjugated or otherwvise unchanged form. Furthermore, the unconjugated enterolactone was below the quantitation limit of the present method in all samples measured. The summary of pharmacokinetic data from the 315 mg/day kinetic subgroup on pharmacokinetic test days 1 and 29 is presented in Table 6.

Table 6. The main pharmakokinetic parameters of unconjugated and total 7-HMR plasma concentration data for the experimental days 1 and 29 in the 315 mg/day dose group

	Day 1		Day 29	
	Unconj. 7-HMR	Total 7-HMR	Unconj. 7-HMR	Total 7-HMR
C_{max} (ng/ml)	50.2 ± 23.8	2032 ± 742	39.2 ± 14.9	2803 ± 699
$t_{1/2}$ (hrs)*	1.4 ± 0.2	1.7 ± 0.6	1.7 ± 0.2	1.6 ± 0.4
AUC_{0-4} (ng h/ml)*	70.8 ± 23.9	4037 ± 848	60.8 ± 17.9	4925 ± 677

* based on calculations of the 0 - 4 hr data after administration of the first daily 105 mg dose, 315 mg/day dose group
The pharmacokinetic analysis did not show evidence of accumulation of HMR or metabolite levels (total or conjugated) with daily dose up to 1350 mg/day.

All adverse events reported during the 4 week study period were mild. According to the investigators assessment, 5 individual events (breath odour, constipation, diarrhea, headache and flatulence) reported in 4 subjects in the HMRLignan™ group were considered possibly treatment-related. Safety laboratory measurements, physical examinations or ECG recordings did not reveal any treatment-related alterations with clinical-toxicological significance during the four weeks' treatment period. HMR was concluded to be safe in human use up to daily dose 1350 mg.

4.3.3 History of use of lignans in the diet

In addition to the clinical studies on HMR, its safety is further corroborated by the history of safe consumption of plant lignans in the diet. Lignans possessing the 2,3-dibenzylbutane carbon skeleton (as with HMR) are ubiquitous in the fiber portion of higher plants. 7-hydroxymatairesinol is present in coniferous trees in considerable amounts. In edible foodstuffs, 7-hydroxymatairesinol is found in *e.g.* sesame seeds[56,57]. Furthermore, closely related plant lignans, *e.g.* matairesinol and secoisolariciresinol diglycoside, are present in considerable amounts in several edible plants[58]. Flaxseed is the richest characterised source of plant lignans. Other considerable lignan sources are whole grain cereal products, *e.g* rye. Matairesinol and secoisolariciresinol are known precursors for enterolactone, and diets enriched with *e.g.* flaxseed or whole grain products have been shown to elevate circulating enterolactone in human subjects[59,60].

The main metabolite of HMR is enterolactone and it is considered in the literature as a phytoestrogen[58,61]. Enterolactone binds to the estrogen receptors with low affinity, and it is also postulated to be the causative agent / bioindicator of the positive health effects associated with intake of lignan containing foodstuffs[58]. Positive disease risk reducing health effects associated with high plasma lignan (enterolactone) concentration have been demonstrated for breast cancer and cardiovascular disease. These associations have been mainly found in epidemiological case-control studies. While the actual mechanism of

action behind the beneficial disease risk reducing effects of lignans remain unresolved, it is possible that a weak antiestrogenic effect by enterolactone mediates them. However, the precursor lignans and their metabolites are strong antioxidants (mainly demonstrated *in vitro*) and that may also represent one mechanism for the abovementioned disease risk reducing effects.

4.4 Synopsis of the toxicologically relevant issues presented in this dossier

Table 7 presents a synopsis of the toxicologically relevant observations of HMR and their calculated safety margins from different exposure related viewpoints compared to the proposed 50 mg maximum recommended daily human dose.

Table 7. Synopsis of Toxicological Observations, Toxicokinetic Data, and Calculations of Margins-of-Safety for Humans Under the Intended Conditions of Use

Effect	Parameter	Animal exposure	Human exposure 50 mg / day	Safety (exposure) factor
Decreased ovary weight, 13 wk study	Dose	160 mg/kg/day	1 mg/kg (50 kg person) 0.71 mg/kg (70 kg person)	160 x 225 x
Decreased ovary weight, 13 wk study	Steady st. plasma enterolactone	62 ng/mL	0.06 ng /mL	>1033 x
Decreased ovary weight, 13 wk study	Steady st. plasma HMR	74 ng/mL	0.59	125 x
Decreased ovary weight, 13 wk study	HMR AUC	1120 h.ng/mL	25 h.ng/mL	45 x
Rat micronucleus, negative result	Dose	2 000 mg / kg	1 mg/kg (50 kg person) 0.71 mg/kg (70 kg person)	> 2000 x > 2816 x
Rat micronucleus, negative result	HMR AUC	26 800 h.ng/mL	25 h.ng /mL	> 1072 x
Rat micronucleus, negative result	HMR Cmax	1147	11	>104 x

In the HMR safety studies in which relevant effects have been noted, the rat exposures have been much higher as compared with the exposures that follow from the maximum suggested daily intake of 50 mg HMR per day (safety margins of mostly between 100- to over 1000-fold). Only in one case was the safety margin below 100 (45-fold for decreased relative ovarian weights in the 13 week study based on comparison of rat and human HMR AUC values). However, this comparison is of minimal relevance, since HMR does not appear to be hormonally (estrogenic or antiestrogenic) active (*i.e.*, the enterolactone comparisons are more relevant to assess safety margins).

Enterolactone is normally present in circulation, with an average population level of approximately 10 to 20 nmol/L (or 3 to 6 ng/mL)[62,63]. This figure refers to total enterolactone (glucuronide and sulphate conjugates and unconjugated forms simultaneously) and it appears that in the general population the levels of unconjugated

enterolactone are below the detection / quantitation limits of common analytical methods. The measurements performed in the general population almost exclusively refer to total rather than unconjugated enterolactone. In our studies we were not able to detect any unconjugated enterolactone in control subjects. In rats fed HMR unconjugated enterolactone represent approximately 3 % of the total circulating enterolactone. Free enterolactone is detected in humans only if very large single doses (> 300 - 600 mg) of precursor lignan (HMR) are administered. In the 13-week study in which rats were fed HMR at 160 mg/kg/day, considerable unconjugated levels of enterolactone were detected at 62 ng/mL, or 208 nmol/L. In human subjects, consumption of HMR at 315 mg/day for 28 days did not result in measurable unconjugated enterolactone (detection limit 0.4 ng/mL). Therefore, it is not likely that HMR consumption at the maximum recommended dose of 50 mg should result in hormonal effects in humans large enough to manifest any considerable alterations in ovaries or other endocrinologically active organs.

The clastogenic effect of HMR in the CHO cells occurs in association with a high level of cytotoxicity, and thereby appears not due to intrinsic activity of HMR itself. This conclusion is supported by the presented cytotoxicity data of HMR indicating that acute toxicity occurs within the same dose range as that at which chromosomal damage is observed. The non-genotoxicity of HMR is also supported by the negative Ames test. Furthermore, the rat micronucleus test with HMR systemic exposure levels of many multiples that of anticipated to occur under the intended conditions of use in humans proved HMR is not mutagenic *in vivo*. Finally, the published data shows that compounds closely related to HMR have also been shown to be non-genotoxic in chromosome aberration tests.

Collectively, a comparative analysis of the toxicological, toxicokinetic, and human bioanalytical data on HMR supports the conclusion that HMRLignan™ can be safely used as dietary supplement product, at a maximum recommended dose of 50 mg / day for adults.

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